



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A01K 67/027, C12N 5/10, A61K 49/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 99/37142</b> <b>(43) International Publication Date:</b> 29 July 1999 (29.07.99)
<b>(21) International Application Number:</b> PCT/DK99/00041 <b>(22) International Filing Date:</b> 27 January 1999 (27.01.99)  <b>(30) Priority Data:</b> 0115/98 27 January 1998 (27.01.98) DK 60/074,170 9 February 1998 (09.02.98) US  <b>(71) Applicant (for all designated States except US):</b> NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> MICHELSEN, Birgitte, Koch [DK/DK]; Nybrovej 252A, DK-2800 Lyngby (DK). CHRISTENSEN, Dorthe, Lennert [DK/DK]; Egilsgade 32, 4. tv., DK-2300 Copenhagen S (DK). JORGENSEN, Trine, Nørgaard [DK/DK]; Brønshøjgårdvej 21, 2. tv., DK-2700 Brønshøj (DK).  <b>(74) Common Representative:</b> NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsvaerd (DK).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> METHOD FOR PRODUCING TRANSGENIC ANIMALS  <b>(57) Abstract</b>  A method for producing a transgenic non-human mammal carrying a transgene encoding an immuno inducible autofluorescent protein, said method comprising chromosomally incorporating a first DNA sequence encoding a cytokin promoter operatively connected to a second DNA sequence encoding said autofluorescent protein, such as Green Fluorescent Protein (GFP) or its enhanced variants (EGFP) into the genome of a non-human mammalian animal.		

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## METHOD FOR PRODUCING TRANSGENIC ANIMALS

A method for producing a transgenic non-human mammal, such as a mouse, carrying a transgene consisting of a cytokine promoter and a gene encoding an autofluorescent protein.

This invention relates to transgenic animals.

Transgenic animals carry a gene which has been introduced into the germline of the animal, or an ancestor of the animal, at an early (usually one-cell) developmental stage. Wagner et al. (1981) P.N.A.S. U.S.A. 78, 5016; and Stewart et al. (1982) Science 217, 1046 describe transgenic mice containing human globin genes. Constantini et al. (1981) Nature 294, 92; and Lacy et al. (1983) Cell 34, 343 describe transgenic mice containing rabbit globin genes. McKnight et al. (1983) Cell 34, 335 describes transgenic mice containing the chicken transferrin gene. Brinster et al. (1983) Nature 306, 332 describes transgenic mice containing a functionally rearranged immunoglobulin gene. Palmiter et al. (1982) Nature 300, 611 describes transgenic mice containing the rat growth hormone gene fused to a heavy metal-inducible metallothionein promoter sequence. Palmiter et al. (1982) Cell 29, 701 describes transgenic mice containing a thymidine kinase gene fused to a metallothionein promoter sequence. Palmiter et al. (1983) Science 222, 809 describes transgenic mice containing the human growth hormone gene fused to a metallothionein promoter sequence. The present invention now provides a method for producing a transgenic non-human mammal carrying a transgene encoding an immuno inducible autofluorescent protein, said method comprising chromosomally incorporating a first DNA sequence encoding a cytokin promoter operatively connected to a second DNA sequence encoding said autofluorescent protein, such as Green Fluorescent Protein (GFP) or its enhanced variants (EGFP) into the genome of a non-human mammalian animal.

We have generated immuno-reporter mice, which carry a transgene consisting of a promoter and a protein-coding gene. Preferably, the promoter is an inducible cy-

tokine promoter, such as the interleukin-2 promoter, the interleukin-4 promoter or the interferon-gamma gene promoter, which drives the expression of these cytokines in particular T lymphocyte subsets. The protein-coding gene encodes an autofluorescent protein, such as GFP (Green Fluorescent Protein) and its related variants, such as EGFP (Enhanced Green Fluorescent Protein).

In a preferred embodiment said first DNA sequence encodes an interleukin promoter, preferably the IL-2, the IL-4 promoter or the interferon-gamma gene promoter.

10 In another preferred embodiment said second DNA sequence encodes an EGFP as disclosed in PCT/DK96/00051.

In another preferred embodiment said non-human mammal is a rodent, such as a mouse.

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In another preferred embodiment said rodent is the bb rat or the nod mouse.

In another preferred embodiment said second DNA sequence encoding GFP or an EGFP is under the control of the cytokin promoter sequence, preferably the IL-2 promoter sequence, the IL-4 promoter sequence, or the interferon-gamma gene promoter.

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In another preferred embodiment said cytokin promoter sequence controlling transcription of said GFP or EGFP gene sequence is inducible.

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In another preferred embodiment said second DNA sequence is fused to said inducible cytokin promoter sequence.

In another preferred embodiment said GFP or EGFP gene is derived from a plasmid, such as the Clontech EGFP plasmid.

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In another preferred embodiment said chromosomal incorporation is at a stage no later than the 8-cell stage.

5 In another preferred embodiment said chromosomal incorporation is at a stage no later than the 1-cell stage.

In another preferred embodiment a linearized plasmid comprising said first and said second DNA sequences are microinjected into a fertilized oocyte.

10 In another preferred embodiment said linearized plasmid is produced by inserting a 518 bp; a 850 bp or a 3000 bp; or a 3448 bp DNA sequence encoding the IL-2 promoter, the IL-4 promoter, or the interferon-gamma gene promoter, respectively, upstream of the EGFP gene and down stream of the SV40 polyAdenosyl sequence of a suitable plasmid carrying the desired GFP or EGFP gene, such as the Clontech  
15 plasmid, to create a linearized plasmid.

In another aspect the invention relates to a method of testing a material suspected of being capable of activating T-lymphocytes which comprises exposing an animal produced according to the method of any one of claims 1 to 13 to said material and  
20 detecting induction of expression of GFP or EGFP as an indication of T-lymphocyte activation.

In another aspect the invention relates to a transgenic non-human mammalian animal whose germ cells and somatic cells contain an inducible GFP or EGFP sequence as a result of chromosomal incorporation into the animal genome, or into the  
25 genome of an ancestor of said animal, said GFP or EGFP gene optionally being further defined according to the invention as described in PCT/DK96/00051.

30 In a preferred embodiment the animal is a rodent, such as the c57bl/6 mouse or an animal which is genetically predisposed to the development of diabetes, such as the bb rat or the nod mouse.

In another preferred embodiment said chromosomal incorporation is at a stage no later than the 8-cell stage.

- 5 In another preferred embodiment said chromosomal incorporation is at a stage no later than the 1-cell stage.

In another aspect the invention relates to a chromosome of an animal as claimed in claim 16, which comprises an EGFP gene as defined in claim 3.

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In another aspect the invention relates to a method of providing a cell culture comprising providing a transgenic non-human mammalian animal as defined in any one of claims 15 to 16 and culturing a somatic cell thereof.

- 15 In another aspect the invention relates to a cell derived from a somatic cell obtained from a transgenic non-human mammalian animal as defined in any one of claims 15 to 18.

In another aspect the invention relates to a non-invasive method of monitoring T-lymphocyte activation wherein a transgenic mouse, a culture of T-lymphocytes or a suspension of T-lymphocytes transfected with a GFP gene operatively connected to a cytokine promoter, such as the IL-2 promoter, the IL-4 promoter, or the interferon-gamma gene promoter, is contacted with a substance suspected of being capable of activating T-lymphocytes and measuring the resulting fluorescence.

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Other features and advantages of the invention will be apparent from the description of the preferred embodiments, and from the claims.

The drawings will now briefly be described.

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Figure 1-6: show plasmid diagrams of promoter-gene constructions according to the invention.

Figure 7: 6 fluorescence images showing 1AD2 (IL-2/EGFP) cells added to primed JAWS (t=0).

Figure 8: 9 fluorescence images showing 1AD2 (IL-2/EGFP) cells added to primed JAWS (t=0) with different concentrations of Der p1.

Figure 9: 1AD2 (IL-2/EGFP) cells added to primed JAWS (at t=0), with 1 mg/ml Der p1. The fluorescence images show the same well at the indicated time points (hrs).

Figure 10: 1 AD2 (IL-2/EGFP) cells added to JAWS (t=0), primed with different doses of Der p1. The intensity was measured at the given time points.

Figure 11: 2BB11 (IL-2/EGFP) cells added to JAWS (t=0), primed with different doses of Der p1. The intensity was measured at the given time points.

Figure 12: 1AD2 (IL-2/EGFP) cells added to primed JAWS (t=0), with 1 mg/ml Der p1 together with different concentrations of Cyclosporin A (CsA). All data were gathered at t=22 hr and are shown relative to primed JAWS without Der p1 (0).

Figure 13: 2BB11 (IL-2/EGFP) cells added to primed JAWS (t=0), with 1 mg/ml Der p1 together with different concentrations of Cyclosporin A (CsA). All data were gathered at t=22 hr and are shown relative to primed JAWS without Der p1 (0).

Figure 14: 1AD2 (IL-2/EGFP) cells added to primed JAWS (t=0), with 0.1 mg/ml Der p1 together with different concentrations of ligands. All data were gathered at t=24 hr and are shown relative to primed JAWS without Der p1 (0).

Figure 15: 1AD2 (IL-2/EGFP) cells added to primed JAWS (t=0), with 1 mg/ml Der p1 together with different concentrations of inhibitors. All data were gathered at t=24 hr and are shown relative to primed JAWS without Der p1 (0).

- 5 Figure 16: is a diagram of fluorescence intensity of 1AD2(IL-44/EGFP) cells added to primed JAWS (t=0) with different concentrations of Der p1 and ionomycin added.

Figure 17: is a diagram of fluorescence intensity of 2BB11(IL-44/EGFP) cells added to primed JAWS (t=0) with different concentrations of Der p1 and ionomycin added.

- 10 Figure 18: is a poster describing a physiologically relevant system comprising antigen-specific stimulation of two T cell hybridoma cell lines by a dendritic cell line.

Autoreactive T cells are the primary mediators of beta-cell destruction occurring in IDDM. The cytokine profile, Th1 or Th2, secreted by these T cells modulate this autoreactive response. Accordingly, we have created a cellular system, which enables the differentiation of stimuli, signals and pathways participating in the determination of T cells to become Th1 or Th2 type at the single cell level. Using imaging, several cellular parameters can be followed dynamically and correlated in multiparameter studies. For instance GFP can be used as a reporter protein of gene activation, while intracellular  $\text{Ca}^{2+}$ , a main cellular signal molecule following TCR activation, can be measured simultaneously. For this purpose the mouse IL-2 (International Immunology Vol 6, No. 2, pp 189-197, F. Brombacher (1993)) and IL-4 promoters have been cloned and fused to the promoterless pEGFP-1 vector. These constructs have been stably transfected into two hybridoma cell lines, 1AD2 and 2BB11, specifically reactive towards the dust mite allergen Der p1. Data on induction of the IL-2 and IL-4 promoter as measured by EGFP fluorescence in the two hybridoma cell lines, 1AD2 and 2BB11 (kindly provided by Dr. J. Lamb, Imp. College, London), are presented. The main focus is on the induction of the IL-2 promoter following stimulation with APCs presenting a specific peptide, or by increasing intracellular calcium. A low constitutive level of GFP is observed in unstimulated cells. A dendritic cell line, JAWS, can be induced to present Der p1 after addition of  $\text{TNF}\alpha$ ,  $\text{IFN}\gamma$  and IL-4.

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Stimulation of the two cell lines with APCs induces a rise in GFP fluorescence, starting after two hrs. and rising to a max. level between 16-24 hrs. The induction is antigen specific. The physiologic stimulation using APCs and antigen, in addition to pathway specific drugs and blocking antibodies enables us to study single cell cytokine gene induction and the signaling pathways involved in single cell determination.

Insulin Dependent Diabetes Mellitus is a T-cell mediated autoimmune disease characterised by  $\beta$ -cell destruction. Some of the events leading to IDDM are thought to depend on cytokines released by infiltrating T lymphocytes in the pancreas.

We want to establish a physiologically relevant *in vitro* system for investigating the antigen-dependent stimulation of cytokine promoters in T cells. For this we have used two T cell hybridoma cell-lines, 1AD2 and 2BB11 as described above. These hybridomas can be stimulated by a dendritic cell line, JAWS (Kindly provided by Dr. V. Mackay, Zymogenetics Institute, Seattle), generated from p53<sup>-/-</sup> C57bl mice and known to express MHC class II molecule; I-A<sup>b</sup>. We quantified cytokine gene activation by RT-PCR, secreted cytokines by ELISA and proliferation by a propidium iodide based assay. At stimulation with antigen, the hybridomas respond by a fast upregulation of c-Rel and concomitant suppression of c-maf mRNA levels. Shortly after, IL-2 and IL-4 mRNA levels are increased, peaking at app. 2hrs, followed by secretion of these cytokines. After another 3 hrs of stimulation, the FasL/Bcl-2 mRNA ratio increases 3-5 fold, suggestive of antigen-induced apoptosis. Thus, these hybridoma lines behave as normal T cells upon antigen stimulation.

To test the validation of the system we fused the IL-2- and IL-4-promoters to EGFP-1. In this system the level of GFP mRNA and the appearance of GFP fluorescence follows the pattern of induction of the endogenous cytokine genes. We therefore conclude that this system can be used to follow the induction of cytokine promoters in living cells.

### Construction of reporter genes

The IL-2 promoter/enhancer element was cloned from NOD mouse chromosomal DNA by PCR and contains the 518 bases upstream of AUG. It was inserted in the promoterless pEGFP-1 vector. The IL-4 construct was made in a similar way.

## 5    **Stable transfection of hybridoma cell lines**

The IL-2/EGFP and IL-4/EGFP constructs were stably transfected into two hybridoma cell lines; 1AD2 and 2BB11, specifically reactive towards the dust mite antigen Der p1. Transfection was done by electroporation.

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## **Activation of hybridoma cells with antigen presenting cells**

A dendritic cell line, JAWS, can be induced to present the dust mite peptide Der p1 in the following way:

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Day 1: JAWS growing in the presence of GM-CSF.

Day 2: Addition of TNF $\alpha$ , IFN $\gamma$  and IL-4.

Day 3: Der p1 added.

Day 4: Cytokines removed and hybridoma cells are added at t=0.

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## **Imaging in 96 well plates**

After addition of hybridoma cells to APCs growing in 96 well plates, the intensity of IL-2/EGFP or IL-4/EGFP was measured in an automated 96 well plate imaging system.

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An image was taken at a preset location in every well and captured with a CCD camera on an inverted microscope with a controlled stage for 96 well plates. For every image the total intensity of the field of view is calculated.

30    The data shown have been made using a 40 x dry objective.

The images shown with 1AD2 (IL-2/EGFP), figs. 1, 2 and 3, clearly illustrate the induction of EGFP viewed at the single cell level. The induction of EGFP can be followed over time in living cells (fig. 3) and is dependent on the dose of Der p1 (fig. 2). The induction can be seen after 3-5 hrs of stimulation with APCs + Der p1 (fig. 3).

- 5 Fig. 3 shows the same field of view over time, but individual cells cannot be followed due to cell mobility.

Figs. 2 and 3 show that the background is high compared to the signal. This is caused by the medium. Both the signal and background varies from well to well depending on cell density, time of image capture etc. Since total intensity of the field has been used as measure in this study (background subtraction cannot be applied because of the high variation), the signal originating from the cells is underestimated. In figs. 4 and 5 the dose and time dependent fluorescence increase is illustrated for both 1AD2 and 2BB11 cells.

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Fig. 8 shows that the induction of EGFP by a substimulatory dose of Der p1 can be potentiated by ionomycin at 0.1  $\mu$ M - 1  $\mu$ M, where no dose dependent response is seen. Ionomycin alone, without stimulation by JAWS, shows a dose dependent response (data not shown). The potentiated response by ionomycin can be further potentiated by PMA, while PMA alone does not have any effect. The response caused by Der p1, ionomycin and PMA can be totally inhibited by 100 nM CsA. This reflects the important role of calcium activated calcineurin, while PKC only has a potentiating role.

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- 25 Figs. 6 and 8 show that the stimulation of 1AD2 and 2BB11 by 1 mg/ml Der p1 can be totally inhibited by CsA at 25 - 50 nM

Fig. 6 shows 1AD2 cells stimulated with 1 mg/ml Der p1. The response can be dose dependently inhibited by genistein, a tyrosine kinase inhibitor. Rapamycin also shows a dose-dependent inhibition of the response. Wortmannin, a PI3-kinase in-

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hibitor, inhibits the response to some extent, although the result is not clear cut. EGTA totally abolishes the response.

Figs. 7 and 8 show 1AD2 and 2BB11 cells with IL-4/EGFP. The response seen with this promoter is much smaller than for IL-2. Only 1AD2 shows a response to Der p1, while both cell types respond to ionomycin. Other studies shows that the IL-4 induction is much smaller than the IL-2 induction. Therefore, the present assay may not be sensitive enough.

In the immuno-reporter mice according to the present invention, the activation of a particular T lymphocyte is accompanied by induction of an autofluorescent reporter, which can be used as a marker for the functional state of that particular cell. Thus, T cell activation (and modulation thereof) can be studied in these mice *in vivo* as well as *in vitro*. The reporter affects neither the function nor the viability of the cells in which it is induced, and can therefore be studied in living cells and animals. Furthermore, activation of the reporter can be monitored by non-invasive methods, allowing studies of living cells, over time, and *in situ* in living animals or organs. Upon activation, cells expressing the reporter gene can be sorted based on the colour of their fluorescence, e.g. by a fluorescence activated cell sorter (FACS). It is therefore possible to sort out the activated cells, e.g. T cells which respond to a particular antigen, on the basis of the functional state they have obtained, which labels the cell with a fluorescent colour. For example, if the activated T cell differentiates along the Th2 pathway and therefore activates its endogenous interleukin-4 gene, that cell will co-activate the interleukin-4 promoter-driven transgene, which might be GFP or an EGFP. On the other hand, if the activated T cell chooses the Th1 pathway, it will activate its endogenous interferon-gamma gene and thereby co-activate the interferon-gamma promoter-driven transgene, such as BFP, the blue EGFP. By use of reporters with different absorption/emission spectra for the different promoter-reporter transgenes, it will thus be possible, upon T cell activation, to distinguish the activation pathway that the T cell undergoes by the colour of the fluorescence that it will develop as a result of reporter-gene activation.

Examples of the use of the immuno-reporter transgenic mice:

The animals of the invention can be used to test a material suspected to activate or  
5 inactivate T cells and to identify materials which can shift the balance between in-  
ducer, suppressor, and cytotoxic responder T cells.

The animals of the invention can also be used to identify pathogenic T cell re-  
sponses, e.g. in animal models of autoimmune diseases. It is possible to raise dia-  
10 betogenic T cell clones from diabetes-prone NOD mice. These clones recognize yet  
unknown target antigens on islet beta-cells. We have now made immuno-reporter  
transgenic mice directly on the NOD genetic background. From these mice, diabeto-  
genic T cell lines can be generated which harbor the promoter-reporter genes, and  
therefore can be used as indicator cells to identify the target antigen(s) as well as  
15 antagonists or modulators which might be anti-diabetogenic.

The animals of the invention can also be used as tester animals to test the effect of  
an immunization and to test the activity and the mechanism of action of immuno-  
modulating drugs and adjuvants and be used to identify materials which can shift the  
20 balance between Th1 and Th2 responder cells. The level (and colour) of T cell  
fluorescence is measured, e.g. in circulating T cells (blood samples at appropriate  
intervals after immunization), or in splenic- or lymphnode- T cells.

The animals of the invention can also be used as donors and/or recipients in T cell  
25 transfers, where the immune response of donor-derived T cells can be studied and  
distinguished from that of the recipient-derived T cells based on their reporter genes.  
This will be an important step forward to identify and study the mechanism of action  
of immunoregulatory (e.g. suppressor-) cell subsets.

30 The animals of the invention can also be used as a source of T cells for cell culture.  
The T cells which are in an activated state (e.g. as a result of viral or bacterial infec-

tion, vaccination/immunization, autoimmune processes, transplantation, exposure to allergen, toxins or immunomodulating drugs) can be sorted by FACS and kept in culture. Such cultures can serve as reporters in *in vitro* screening assays to identify the activating agent (e.g. peptide, drug, virus) as well as antagonists, suppressives and modulators, and be used to identify materials which can shift the balance between Th1 and Th2 responder cells.

The animals of the invention can also be used as a source of antigen specific T cell clones, which can be kept in culture for extended ( or indefinite) periods of time, provided that they are frequently restimulated. Pathological (e.g. diabetogenic, in case the transgene is carried by a diabetes-prone animal) T cell clones from these mice can be used to identify the antigenic peptide which is recognized by their T cell receptor (TCR), because only that peptide will activate the T cell reporter gene via the TCR. Such T cell clones can also be used in *in vitro* screening assays to identify antagonists, suppressives and modulators.

The animals of the invention can also be used to trace the site and the state of activation of Th1- and Th2- cells *in situ* on tissue sections or in whole animals in response to a given stimulus (e.g. antigen priming, infection, autoimmunity, allergy, transplantation, intoxication, inflammation). Preferably, the transgene is expressed in a tissue-specific manner which demonstrates a greater amount of expression in one tissue, such as T cells and basophilic cells, as opposed to one or more second tissues in the transgenic non-human mammal.

The animals of the invention can also be used to identify sites of cytokine gene activation, and pathologies associated with it, in developing bone marrow precursor cells and during maturation of progenitor T cells in the thymus.

Throughout this description the terms GFP and EGFP refers to Green Fluorescent Protein, preferably as occurring in the jelly fish *Aequorea victoria*, and to Enhanced Green Fluorescent Protein as disclosed in PCT/DK96/00051, respectively. Especially

preferred herein are the novel fluorescent proteins F64L-GFP, F64L-Y66H-GFP (Blue Fluorescent Protein or BFP) and F64L-S65T-GFP that result in a cellular fluorescence far exceeding the cellular fluorescence from cells expressing the parent proteins, i.e. GFP, the blue variant Y66H-GFP (wild-type BFP) and the S65T-GFP variant, respectively. The novel fluorescent proteins (EGFP) exhibit high fluorescence in cells expressing them when said cells are incubated at a temperature of 30°C or above, preferably at a temperature of from 32°C to 39°C, more preferably at a temperature of from 35°C to 38°C, and most preferably at a temperature of about 37°C. It is known that fluorescence in wild-type GFP is due to the presence of a chromophore, which is generated by cyclisation and oxidation of the SYG at position 65-67 in the predicted primary amino acid sequence and presumably by the same reasoning of the SHG sequence and other GFP analogues at positions 65-67, cf. Heim et al. (1994). A substitution, of the F amino acid residue at position 1 preceding the S of the SYG or SHG chromophore or the T of the THG chromophore, *in casu* position 64 in the predicted primary amino acid sequence, results in a substantial increase of fluorescence intensity apparently without shifting the excitation and emission wavelengths. This increase is remarkable for the blue variant Y66H-GFP, which hitherto has not been useful in biological systems because of its weak fluorescence. As EGFP's The F64L, F64I, F64V, F64A, and F64G substitutions are preferred, but other mutations, e.g. deletions, insertions, or posttranslational modifications immediately preceding the chromophore are also included in the term EGFP as used herein, provided that they result in improved fluorescence properties of the various fluorescent proteins. It should be noted that extensive deletions may result in loss of the fluorescent properties of GFP. It has been shown, that only one residue can be sacrificed from the amino terminus and less than 10 or 15 from the carboxyl terminus before fluorescence is lost, cf. Cubitt et al. TIBS Vol. 20 (11), pp. 448-456, November 1995. Thus, EGFP relates to a fluorescent protein derived from *Aequorea* Green Fluorescent Protein (GFP) or any functional analogue thereof, wherein the amino acid in position 1 upstream from the chromophore has been mutated to provide an increase of fluorescence intensity when the fluorescent protein of the invention is expressed in cells. Said mutation also results in a significant increase of the intensity of the

fluorescent signal from cells expressing the mutated GFP and incubated at 30°C or above 30°C, preferably at about 37°C, compared to the prior art GFP variants.

### Detailed description of the invention

5 We have generated the following reporter-genes:

The mouse IL-2 promoter/enhancer element (518 basepairs upstream of the AUG) linked to EGFP. NOD mouse genomic DNA was used as template for a PCR reaction using the primers 5'-GGGGATCCTGCAGGACTTGAGGTCAGTGTGAGG-3' and 5'-  
10 GGAAGCTTTAGGAGGTAAACCATCTCGAAACGG-3' in the following cycles: cycle 1: 95°C 90 sec; 45°C 45 sec; 72° 60 sec. Cycle 2-26: 93,5°C 45 sec; 45°C 45 sec; 72°C 60 sec. Cycle 27: 72°C 300 sec. The resulting 521bp product was digested with BamHI and HindIII and ligated into the vector pEGFP-1 (Clontech)(fig. 1) that had been linearized with BamHI and HindIII. The ligated material was used to trans-  
15 form E.coli K803 and transformants selected on LB agar plates with 30mg/ml kanamycin. One colony was found to contain the desired construct (pEGFP-IL-2)(fig. 2).

Same IL-2 promoter/enhancer element linked to EBFP. IL-2-EGFP was cut with BamHI and HindIII and the 521bp fragment containing the IL-2 promoter sequence  
20 was purified by cutting out of an agarosegel. The fragment was ligated into the BamHI / HindIII sites of pEBFP (Clontech)(fig. 3) and used to transform E.coli INVaF' (Invitrogen). Transformants were selected on 50mg/ml ampicillin. One colony was found to contain the desired construct (pEBFP-IL-2)(fig. 4).

25 The mouse IL-4 promoter/enhancer element (850 basepairs and 3kb upstream of transcription initiation site, which lacks the intron 1 element needed for mast cell expression) linked to EGFP (pEGFP-IL-4-801 and pEGFP-IL-4-3kb, respectively). The 850 bp IL-4 promoter fragment was excised from pSV0-801 (Todd, M.D. et al., J.Exp.Med. (1993) 177:1663-74) with HindIII and ligated into pEGFP-1 linearized  
30 with HindIII. The 3 kb IL-4 promoter fragment was excised from pSV0-3kb (ref.) by partial digestion with HindIII. The 3 kb IL-4 promoter fragment was purified from

contaminating fragments by cutting out of an agarosegel. This fragment was then ligated into the HindIII site of pEGFP. IL-4-3kb-EGFP was used to prepare a linearized promoter-reporter fragment for generation of transgenic mice by digestion with XhoI and AflII. The 4,2 kb fragment was purified by cutting out of an agarosegel.

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The 4,2 kb IL-4-3kb-EGFP fragment was used to generate transgenic mice. We now have one founder NOD mouse and its first litter (pups, 3 positive) carrying the IL-4-EGFP transgene.

10 pEBFP-IFNg(fig. 5) was made by excision of the mouse interferon-gamma promoter/enhancer element (3448 basepairs upstream from transcription initiation site) from the plasmid -3441/CAT (Fox, H.S: et al., J.Immunol. (1991) 146:4362-67) with XmaI and BamHI and ligation into the XmaI / BamHI sites of a modified pEBFP vector (pEBFP-5'mod, fig. 6). To produce pEBFP-5'mod, a linker was made by combining  
15 100 pmoles of the oligonucleotides 5'-AGCTTCCCGGGGAATTCCGGATCCA-3' and 5'-CCGGTGGATCCGAATTCCCCGGGA-3' in a total volume of 25ml PCR buffer (Dynazyme) and placed in a thermal (PCR-) cyclor at 95°C for 2 min followed by 30°C for 1 sec. The linker was kept on ice until 1ml was removed and ligated to 100ng pEBFP-1 linearized with HindIII and AgeI. Ligated material was used to  
20 transform E.coli XL1-blue mrf<sup>r</sup> (Stratagene). Transformants were selected on LB agar plates containing 50mg/ml ampicillin. One clone was obtained which contained a single copy of the linker fragment, evidenced by a PCR reaction with the primers 5'-AAAGCGGGCAGTGAGCGCA-3' and 5'-TGAACAGCTCCTCGCCCTT-3' which in the original pEBFP-1 plasmid gives rise to a 234bp product, but in pEBFP-5'mod gives  
25 a 217 bp product.

To test the concept, we have expressed the IL-2-EGFP, IL-4-EGFP, IL-2-BFP and IL-4 BFP reporter genes in T hybridoma lines (1AD2 and 2BB11 from Nanna Kristensen and Jonathan Lamb, recognizing dust-mite allergen der p 1). Transfection  
30 was by electroporation, where  $10^5$  cells / 200ml PBS in a 2mm wide cuvette was placed on ice for 20 min. Plasmid DNA was added, 30mg uncut as well as 30mg that

had been linearized with BglII. The sample was electroporated at 5mF and 210-230V, with an internal resistance of 13V and time constants of app. 0,5 msec. Transfectants were selected after 24 hrs by addition of 1mg/ml G418 to the culture medium, which after 1-2 weeks was raised to 2,3mg/ml.

5  
EGFP (or EBFP, from now only the EGFP data will be mentioned) fluorescence was activated by ionomycin stimulation or by an antigen-presentation assay encompassing a dendritic cell line (JAWS from Vivian MacKay, Zymogenetics) prestimulated with cytokines (GM-CSF, IL-4, TNFa) and coated with the der p 1 peptide. Both assays showed induction of EGFP from undetectable levels before stimulation to high levels in response to stimulation. The kinetics of EGFP mRNA induction paralleled that of the endogenous cytokine mRNA (IL-2 mRNA for IL-2-EGFP and IL-4 for IL-4-EGFP). The fluorescence could be quantified by 1) video imaging microscopy, 2) fluorescence activated cell scanning (FACScan) and 3) a multiwell plate-reader for fluorescence (Victor form Wallac). The appearance of EGFP fluorescence was a marker for cytokine gene induction, cytokine release and antigen-induced cell death in these hybridoma cells. We therefore conclude that these reporter genes can be used as markers for the events that occur when a T cell responds to stimulation with antigen, or when a stimulus is given which bypasses the T cell receptor (e.g. ionomycin). Note that we have also performed the assay in the presence of immunosuppressive agents (e.g. CyclosporinA) and other inhibitors of the T cell receptor signalling cascade (Wortmannin, genistein, rapamycin, EGTA), which inhibited EGFP production in a dose-dependent manner down to background levels. We can therefore also conclude, that we have generated a simple model to study the action of immunosuppressants and the steps at which they interfere with the intracellular signal pathways.

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## CLAIMS

1. A method for producing a transgenic non-human mammal carrying a transgene encoding an immuno inducible autofluorescent protein, said method comprising chromosomally incorporating a first DNA sequence encoding a cytokine promoter  
5 operatively connected to a second DNA sequence encoding said autofluorescent protein, such as Green Fluorescent Protein (GFP) or its enhanced variants (EGFP) into the genome of a non-human mammalian animal.
2. The method according to claim 1 wherein said first DNA sequence encodes an interleukin promoter, such as the IL-2, the IL-4 promoter or the interferon-gamma  
10 gene promoter.
3. The method according to any of claims 1 - 2, wherein said second DNA sequence encodes an EGFP as disclosed in PCT/DK96/00051, preferably F64L-GFP, F64L-Y66H-GFP (Blue Fluorescent Protein or BFP) and F64L-S65T-GFP.
4. The method of any of claims 1 - 3, wherein said non-human mammal is a rodent,  
15 such as a mouse.
5. The method according to the preceding claim wherein said rodent is the bb rat or the nod mouse.
6. The method according to any of claims 1 - 5, wherein transcription of said second DNA sequence encoding GFP or an EGFP is under the control of the cytokine  
20 promoter sequence.
7. The method according to claim 6, wherein said cytokine promoter sequence controlling transcription of said GFP or EGFP gene sequence is inducible.
8. The method as claimed in any one of claims 1 to 7, wherein said second DNA sequence is fused to said inducible cytokine promoter sequence.
- 25 9. The method as claimed in any one of claims 1 to 8, wherein said GFP or EGFP gene is derived from a plasmid, such as the Promega EGFP plasmid.
10. The method as claimed in any one of claims 1 to 9, wherein said chromosomal incorporation is at a stage no later than the 8-cell stage.
- 30 11. The method as claimed in any one of claims 1 to 10, wherein said chromosomal incorporation is at a stage no later than the 1-cell stage.

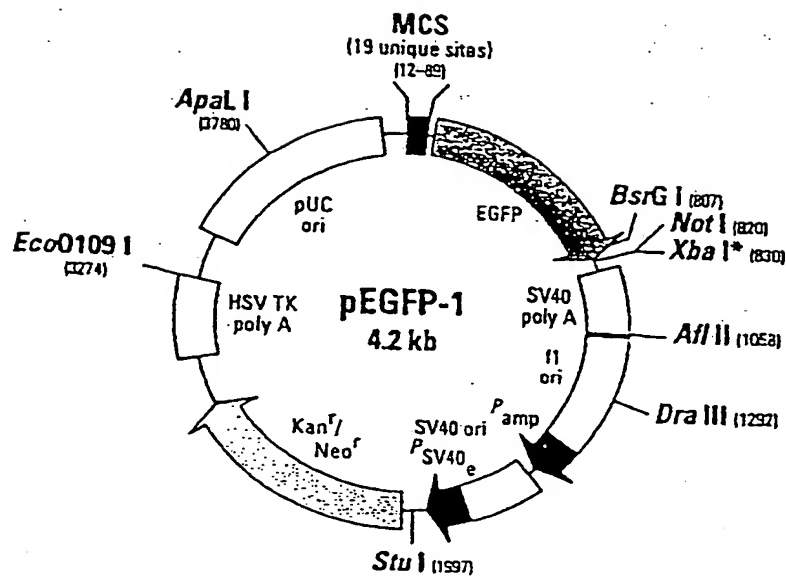
12. The method as claimed in claim 1 which comprises microinjecting into a fertilized oocyte a linearized plasmid comprising said first and said second DNA sequences.
13. The method according to the preceding claim wherein said linearized plasmid is produced by inserting a 518 bp DNA sequence encoding the IL-2 promoter, or a 3000 bp or a 850 bp DNA sequence encoding the IL-4 promoter, or a 3448 bp DNA sequence encoding the interferon-gamma gene promoter up stream of the EGFP gene and down stream of the SV40 polyAdenosyl sequence of the Clontech plasmid pEGFP-1 to create a linearized plasmid.
14. A method of testing a material suspected of being capable of activating T-lymphocytes which comprises exposing an animal produced according to the method of any one of claims 1 to 13 to said material and detecting induction of expression of GFP or EGFP as an indication of T-lymphocyte activation.
15. A transgenic non-human mammalian animal whose germ cells and somatic cells contain an inducible GFP or EGFP sequence as a result of chromosomal incorporation into the animal genome, or into the genome of an ancestor of said animal, said GFP or EGFP gene optionally being further defined according to claim 3.
16. An animal as claimed in claim 15 which is a rodent, such as the c57bl/6 mouse or a mouse which is genetically predisposed to the development of diabetes, such as the NOD mouse.
17. An animal as claimed in claim 15 or claim 16 wherein said chromosomal incorporation is at a stage no later than the 8-cell stage.
18. An animal as claimed in claim 15 or claim 16 wherein said chromosomal incorporation is at a stage no later than the 1-cell stage.
19. A chromosome of an animal as claimed in claim 15, which comprises an EGFP gene as defined in claim 3.
20. A method of providing a cell culture comprising providing a transgenic non-human mammalian animal as defined in any one of claims 15 to 18 and culturing a somatic cell thereof.
21. A cell derived from a somatic cell obtained from a transgenic non-human mammalian animal as defined in any one of claims 15 to 18.

22. A non-invasive method of monitoring T-lymphocyte activation wherein a transgenic mouse, a culture of T-lymphocytes or a suspension of T-lymphocytes transfected with a GFP gene operatively connected to a cytokine promoter, such as the IL-2 promoter, the IL-4 promoter, or the interferon-gamma gene promoter, is contacted with a substance suspected of being capable of activating T-lymphocytes and measuring the resulting fluorescence.

23. A non-invasive method of monitoring T-lymphocyte inactivation or suppression wherein a transgenic mouse, a culture of T-lymphocytes or a suspension of T-lymphocytes transfected with a GFP gene operatively connected to a cytokine promoter, such as the IL-2 promoter, the IL-4 promoter, or the interferon-gamma gene promoter, is contacted with a substance suspected of being capable of suppressing T-lymphocytes and measuring the resulting fluorescence.

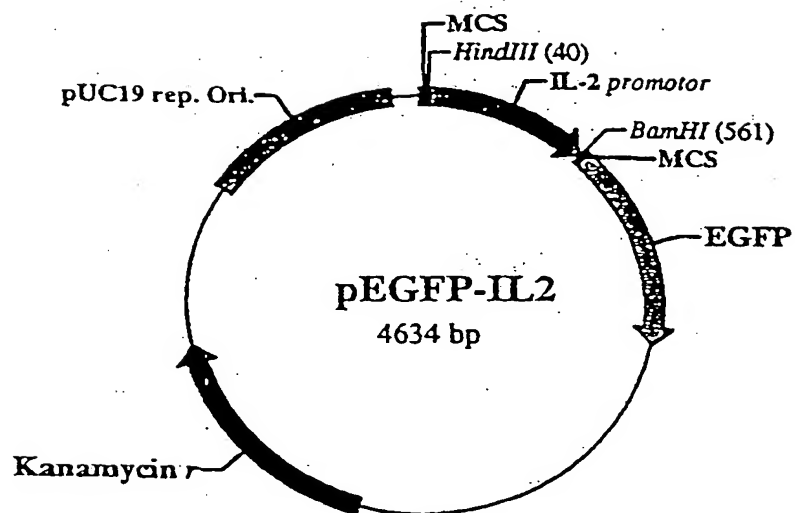
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Figure 1



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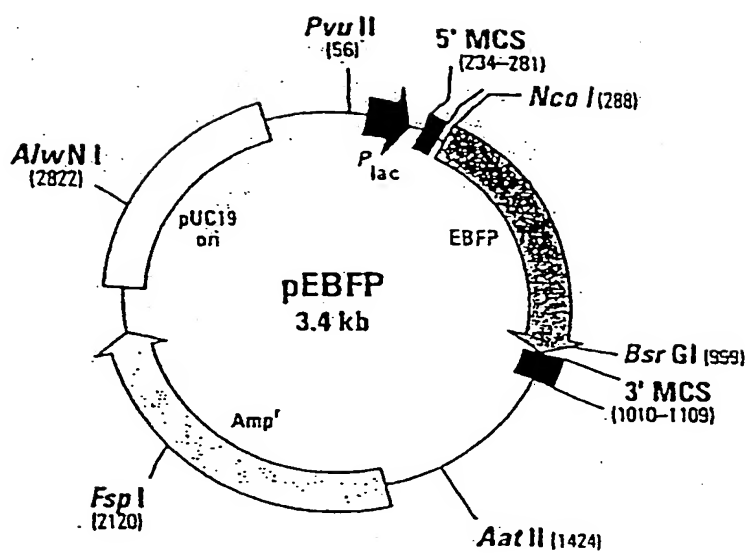
Figure 2



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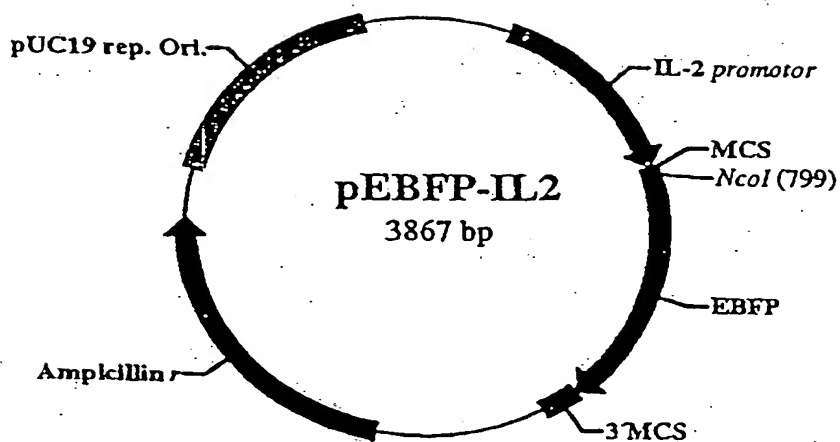
Figure 3



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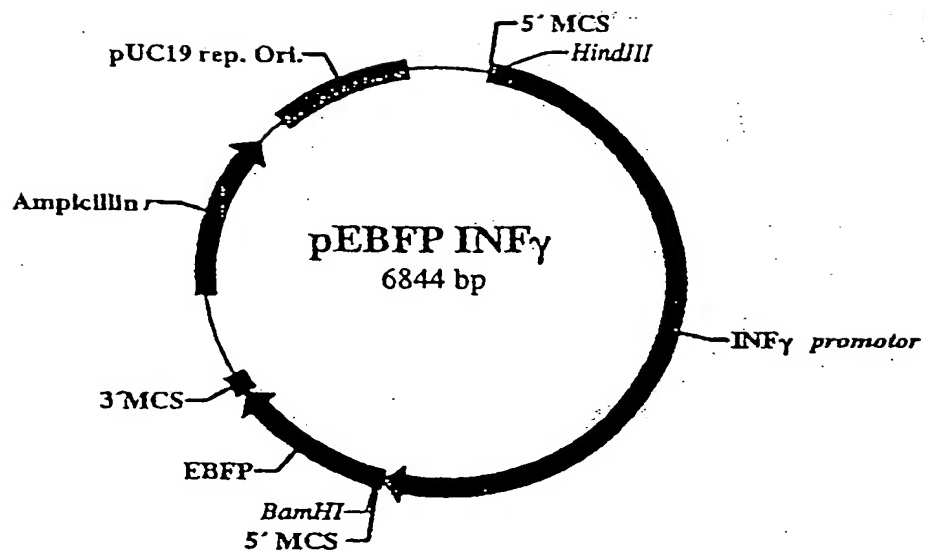
Figure 4



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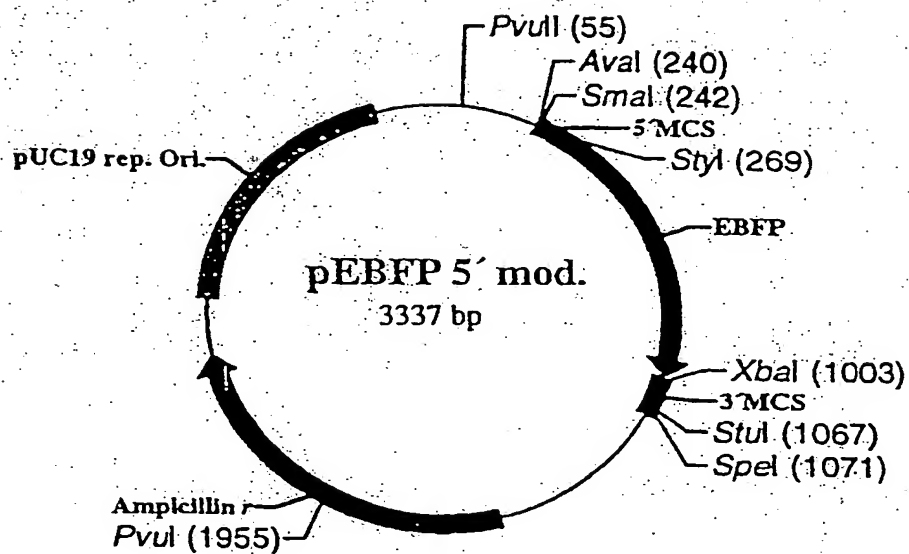
Figure 5



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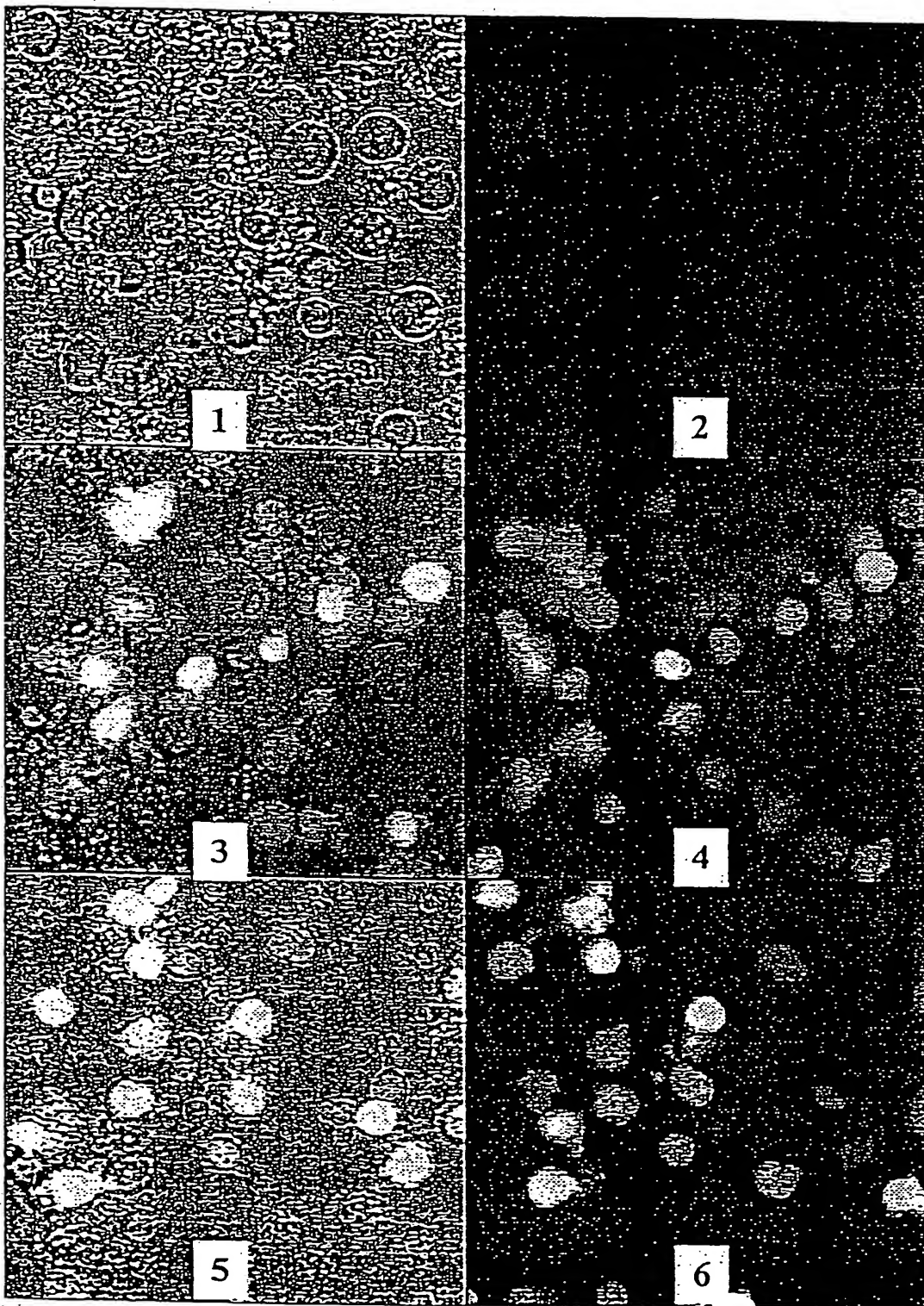
Figure 6



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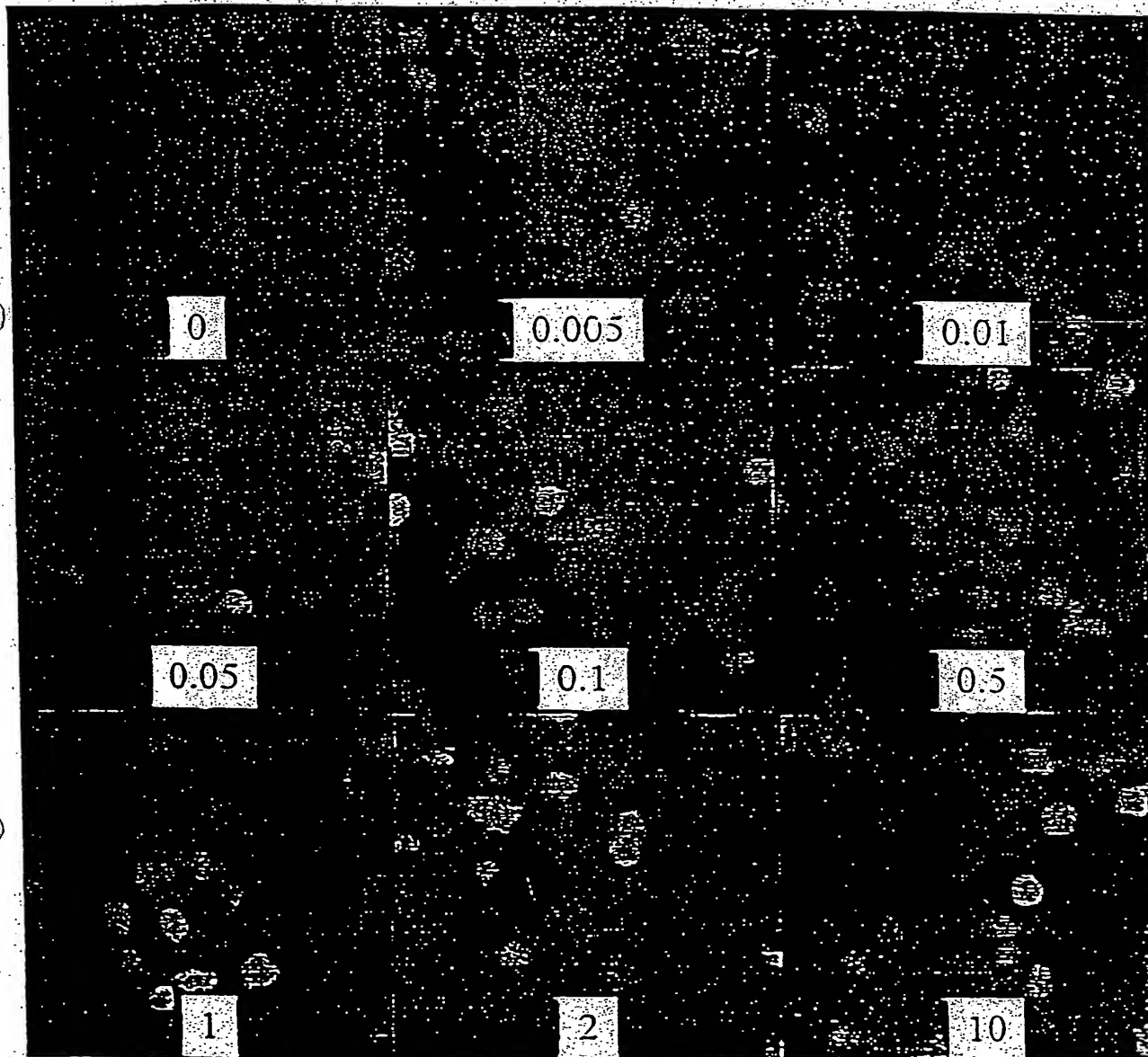
Figure 7



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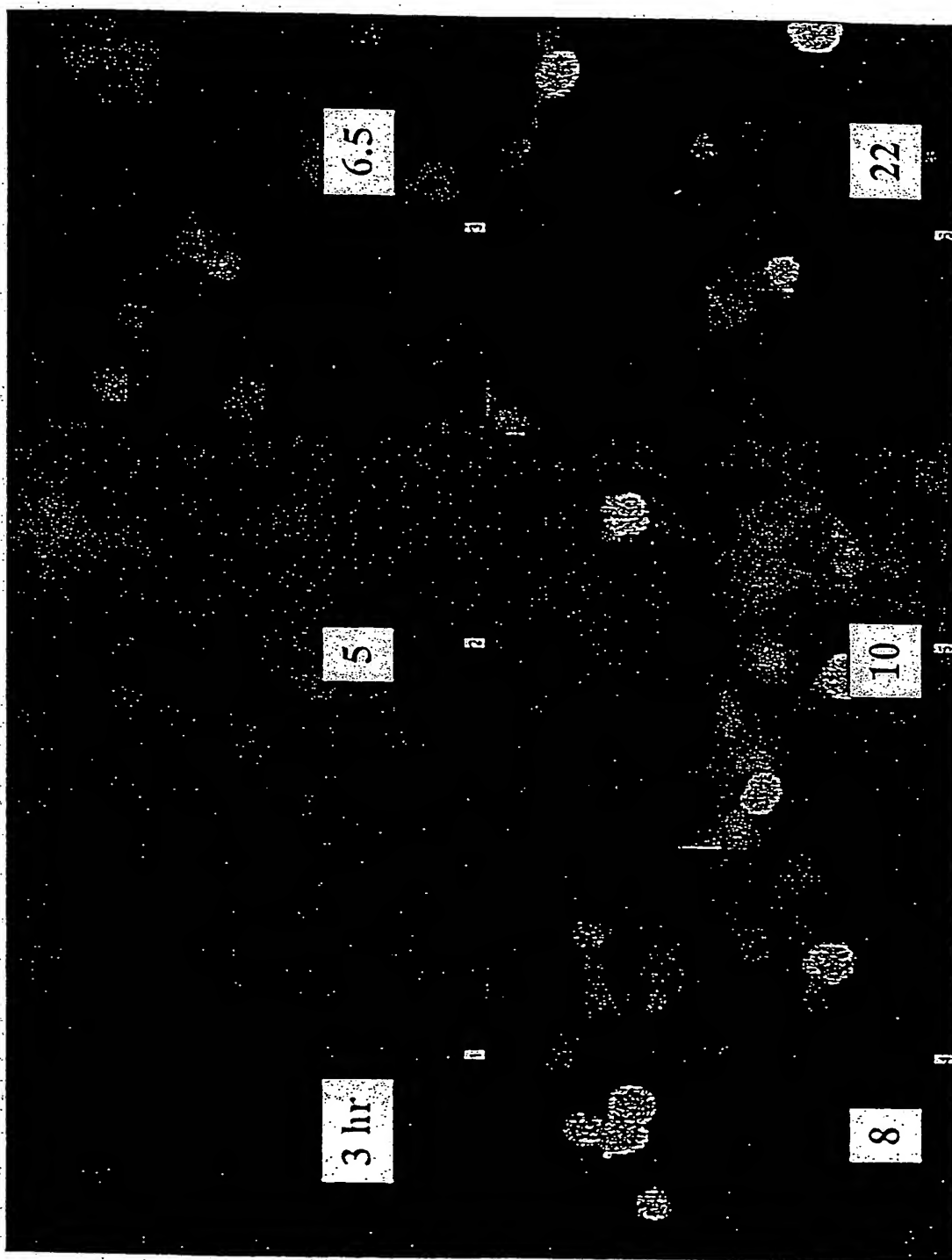
Figure 8



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Figure 9

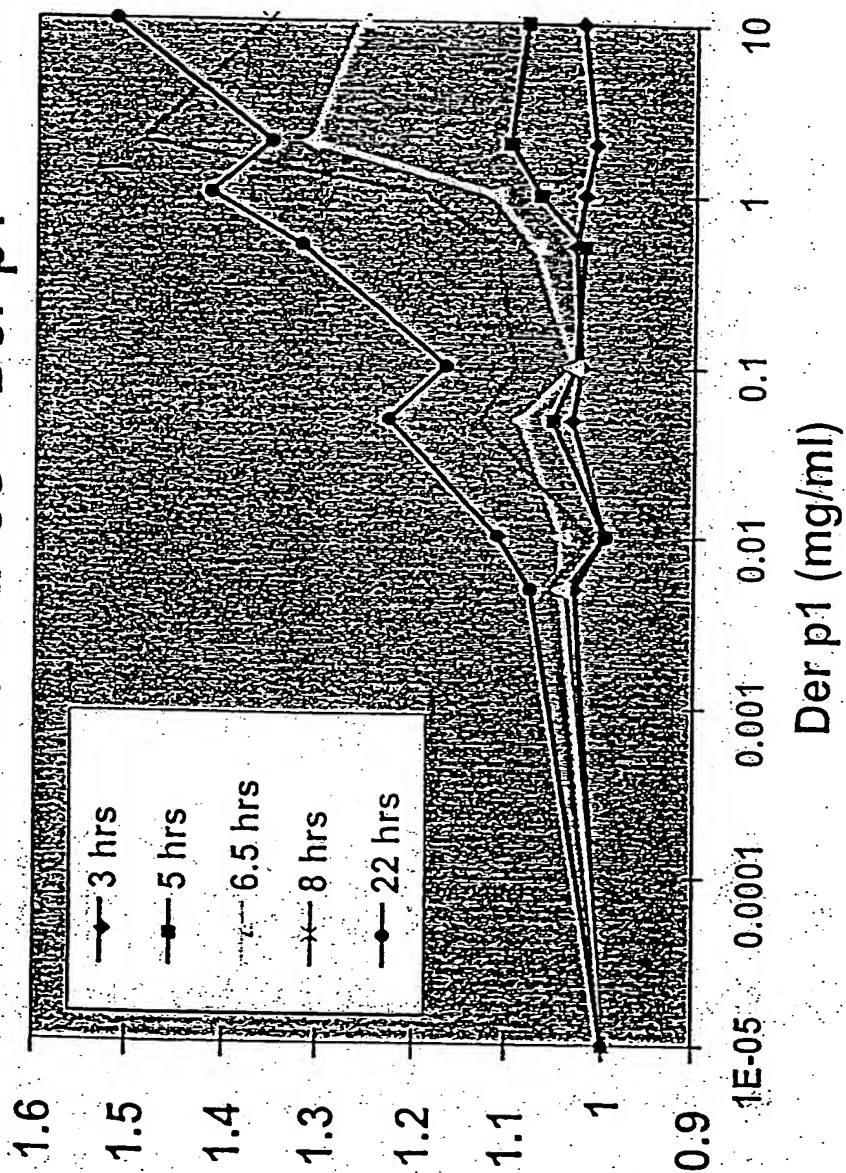


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**1AD2 (IL-2/EGFP).  
Stimulated with APCs + Der p1**

Figure 10

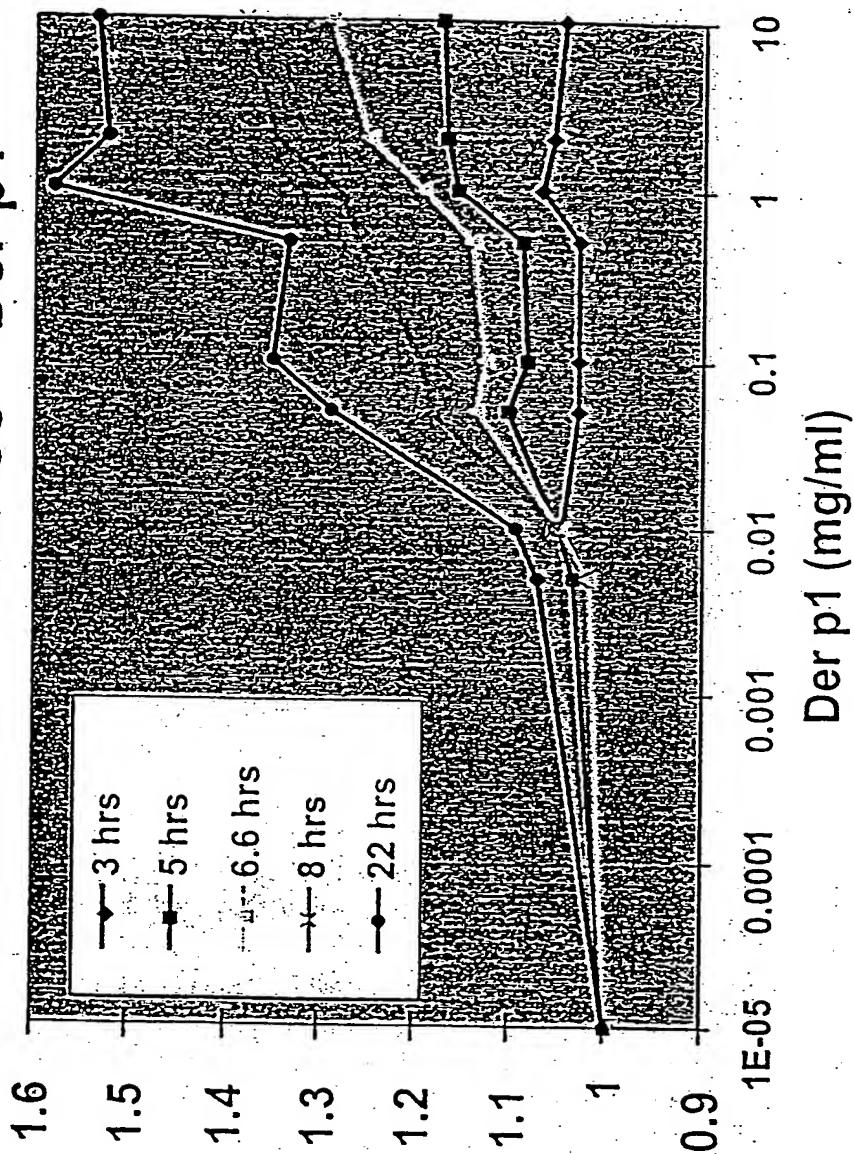


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# 2BB11 (IL-2/EGFP). Stimulated with APCs + Der p1

Figure 11

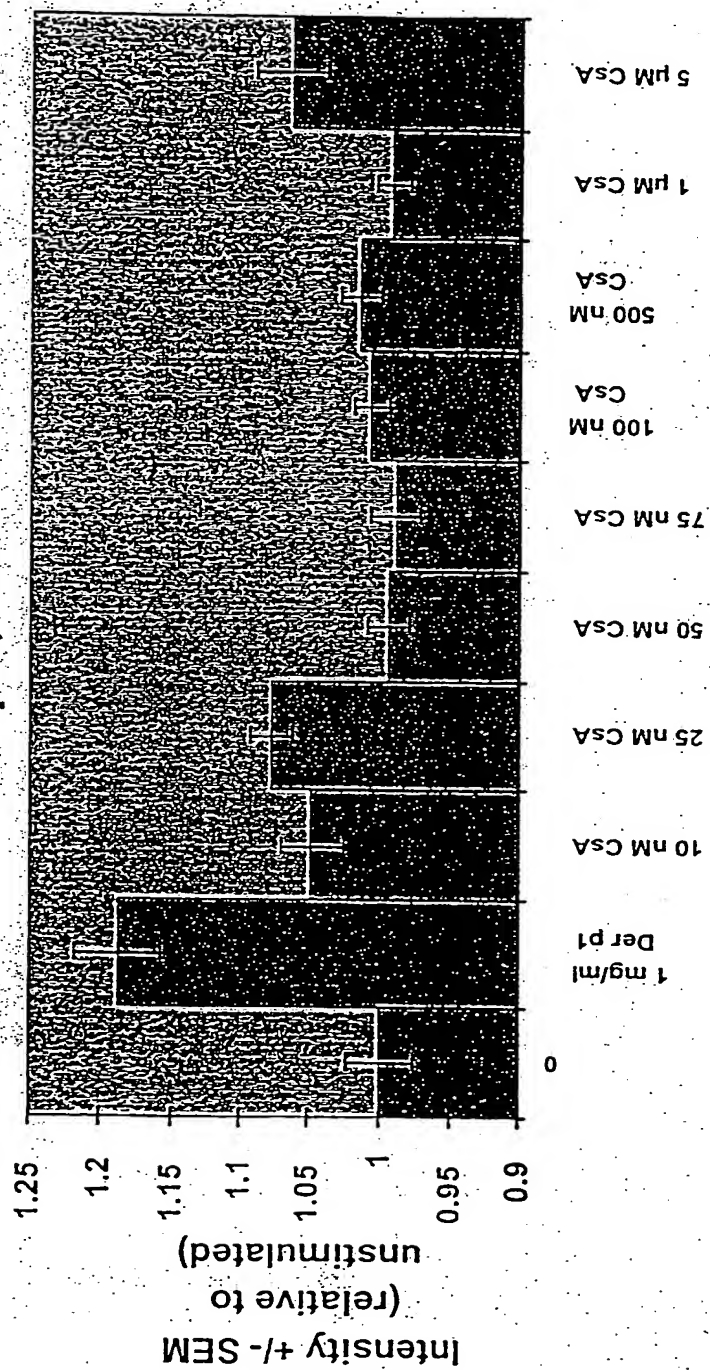


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**1AD2 (IL-2/EGFP).  
Stimulated with APCs +1 mg/ml  
Der p1, and CsA**

Figure 12

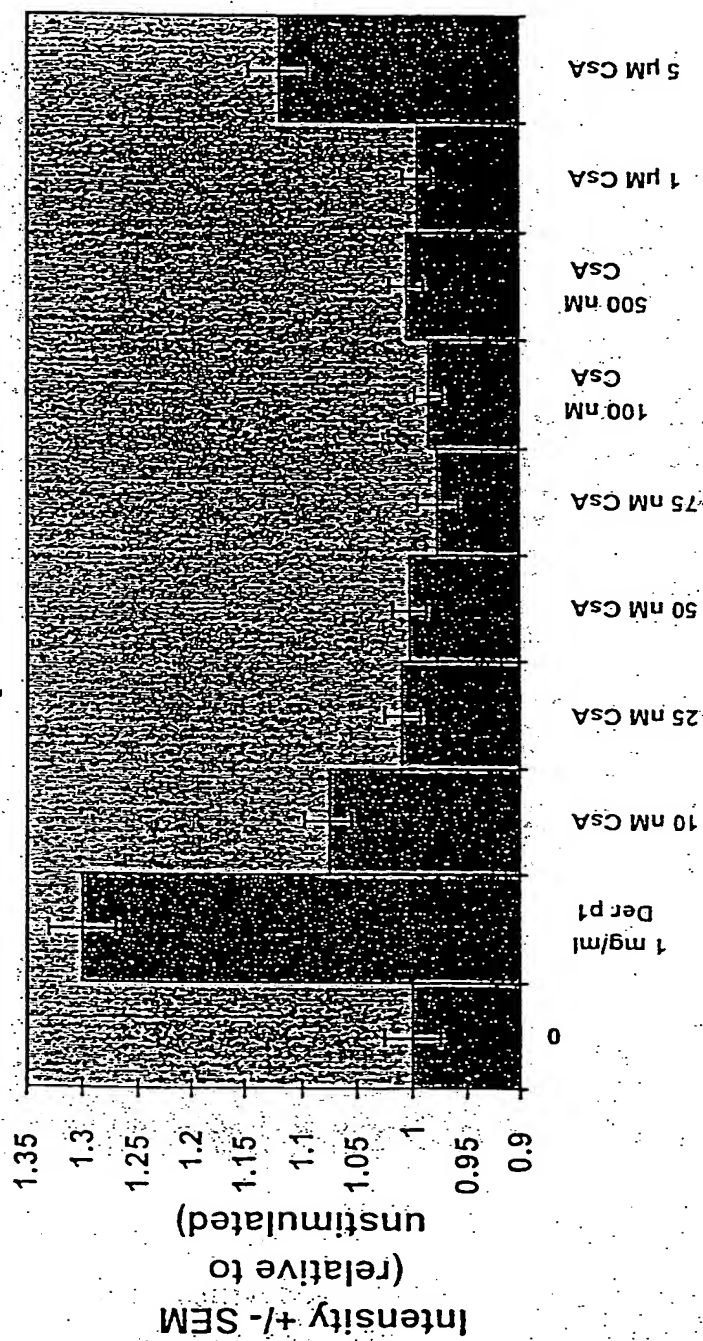


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**2BB11 (IL-2/EGFP).  
Stimulated with APCs +1 mg/ml  
Der p1, and CsA**

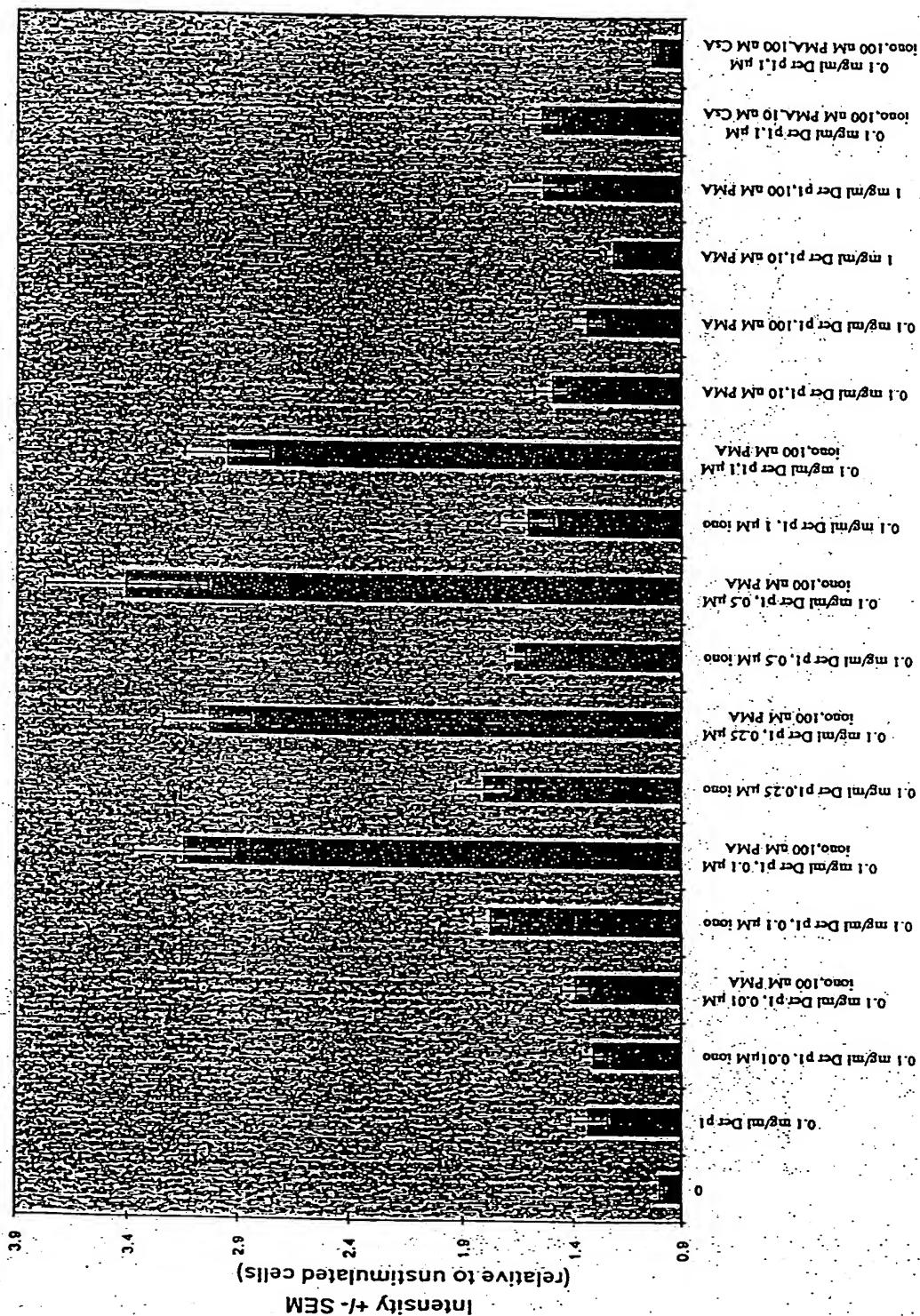
Figure 13



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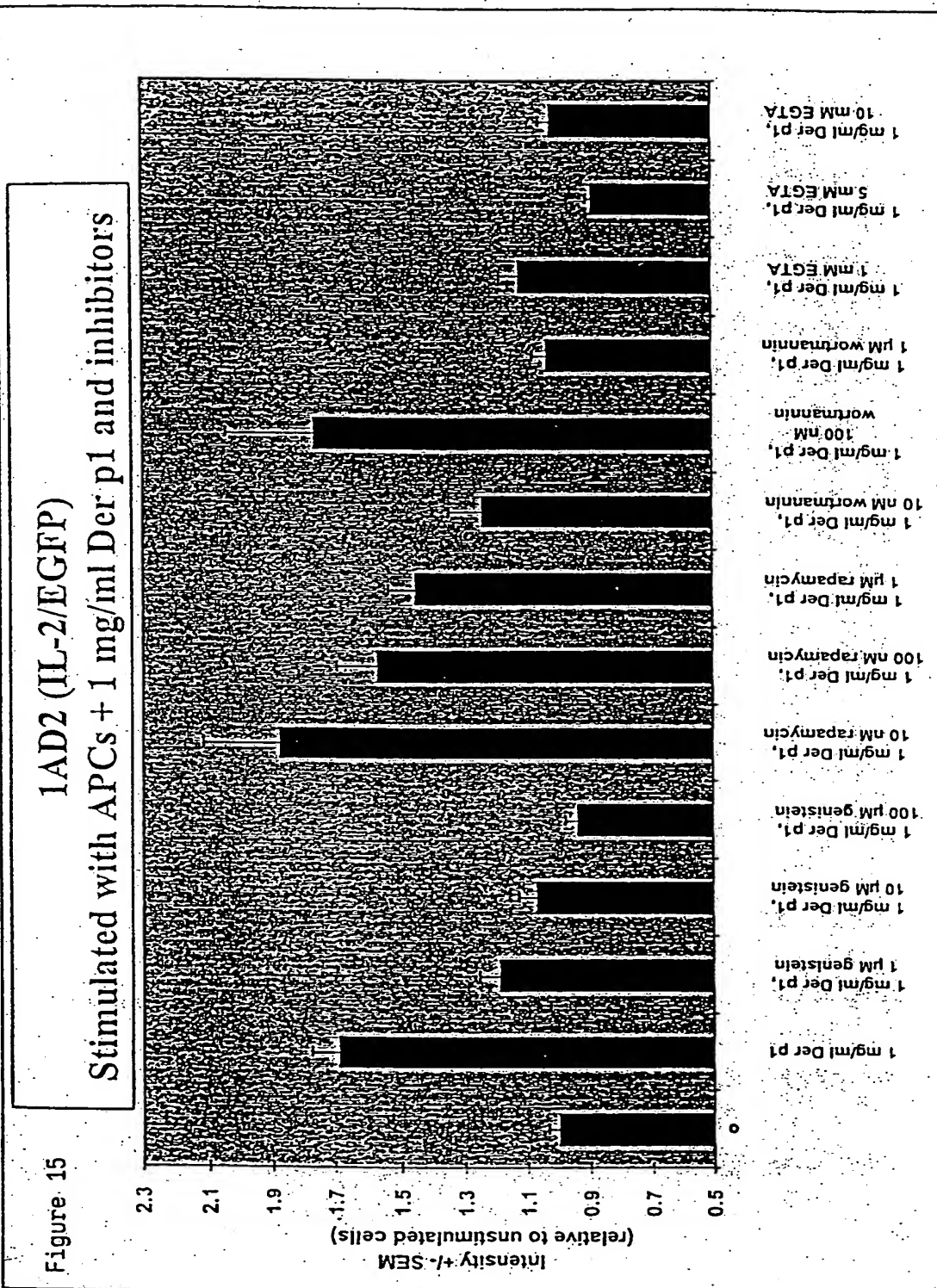
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Figure 14  
1AD2 (IL-2/EGFP).  
Stimulated with APCs + 0.1 mg/ml Der p1, ionomycin, PMA, and  
inhibited with CsA



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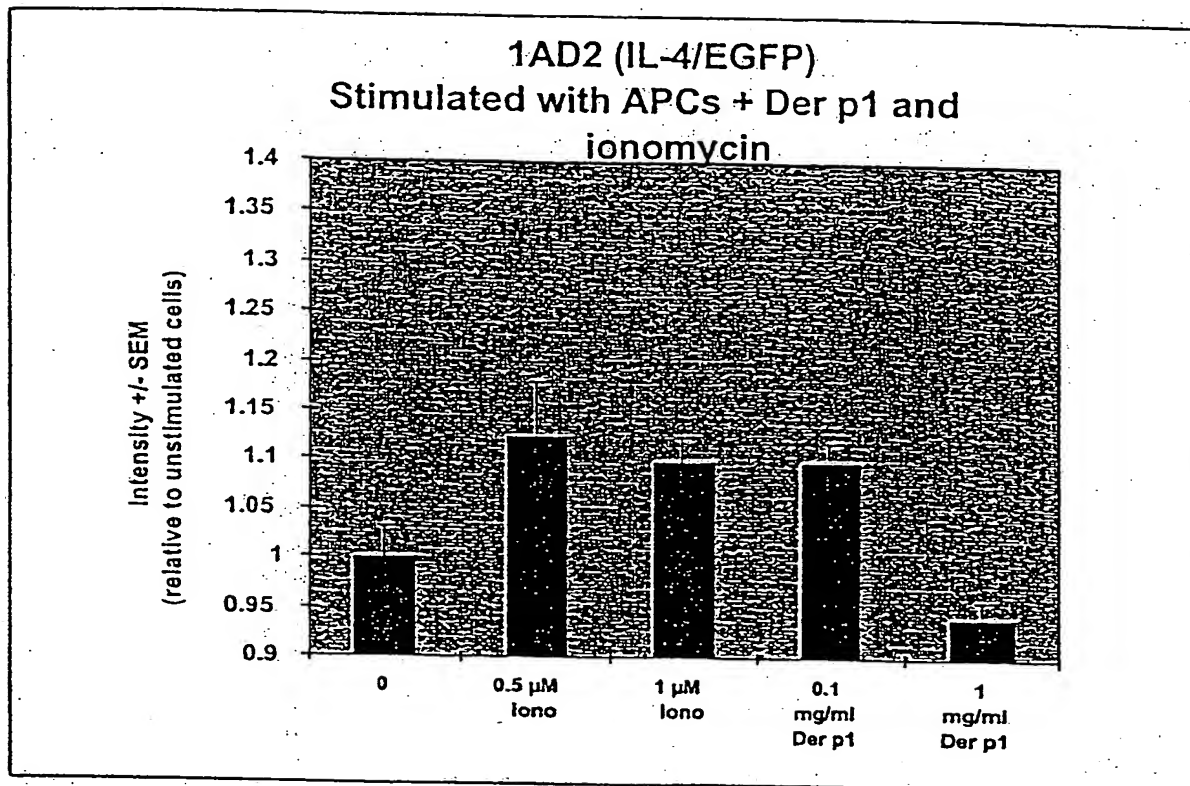
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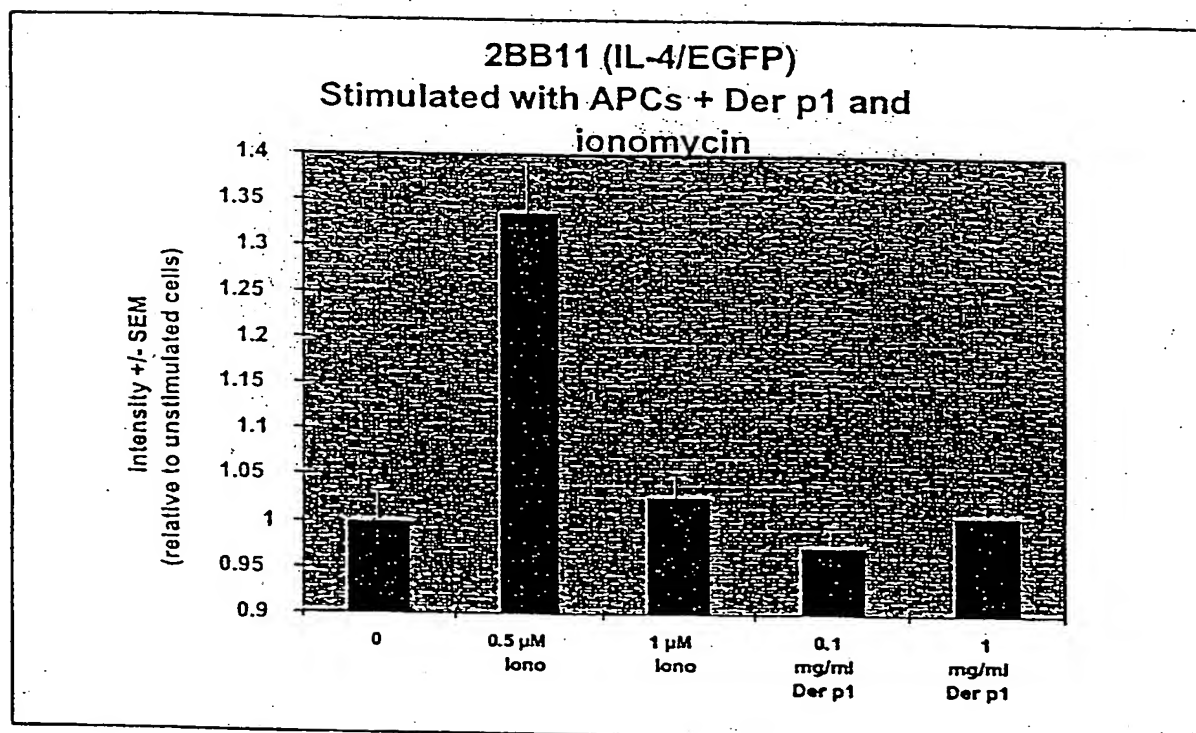
Figure 16



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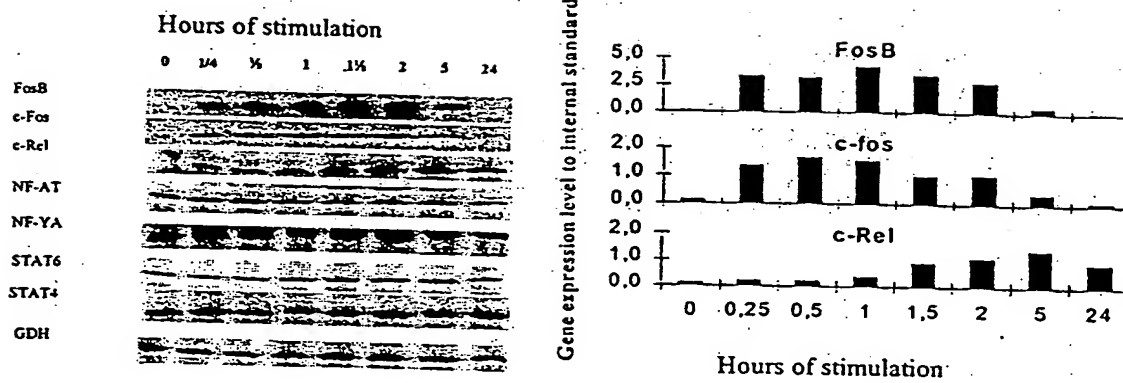
Figure 17



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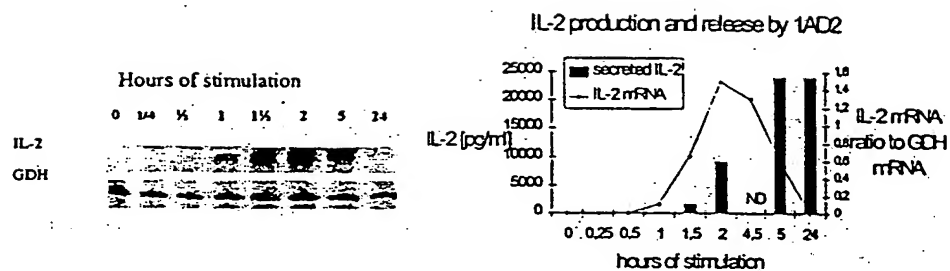
Figure 18



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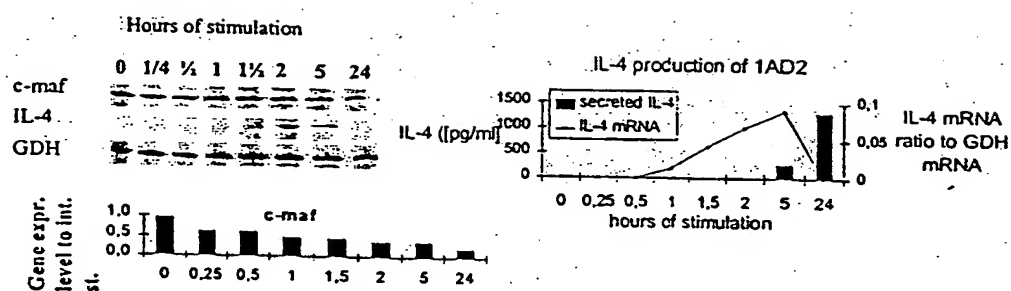
Figure 19



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Figure 20



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Figure 21A

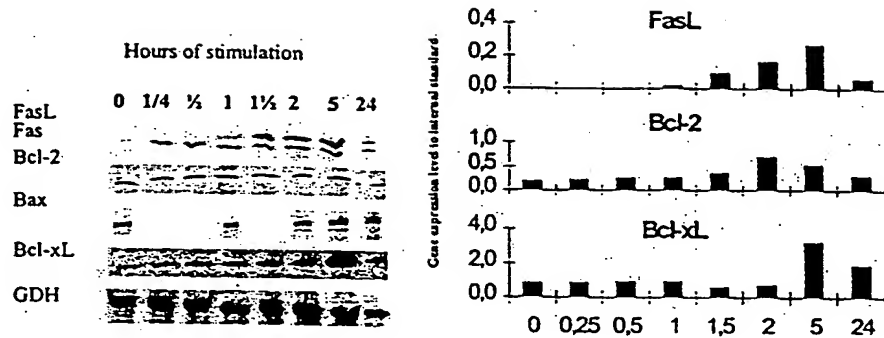
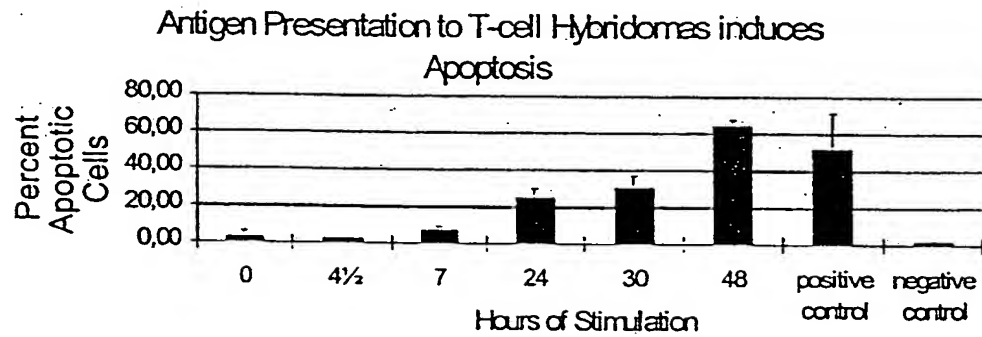


Figure 21B



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Figure 22A

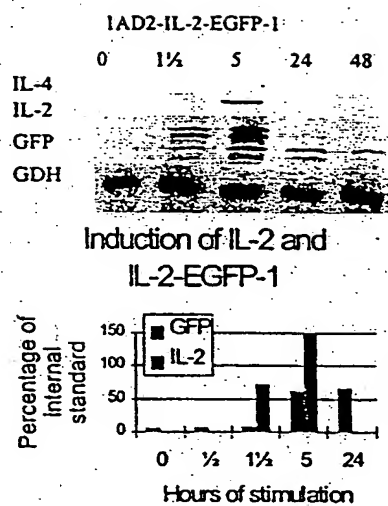
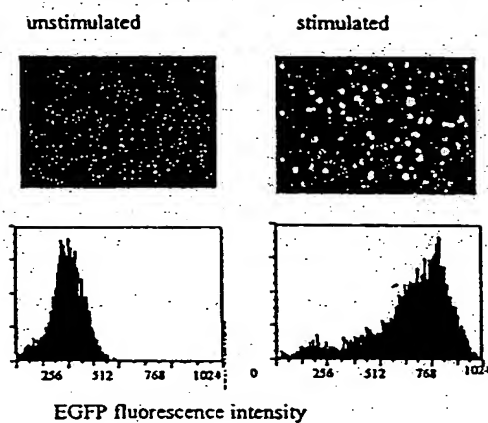


Figure 22B



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# 1

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 99/00041

### A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A01K 67/027, C12N 5/10, A61K 49/00

According to International Patent Classification (IPC) or to both national classification and IPC

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A01K, C12N, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Immunity, Volume 9, August 1998, Mayumi Naramura, et al, "Mice with a Fluorescent Marker for Interleukin 2 Gene Activation" page 209 - page 216	1-23
X	WO 9718841 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY), 29 May 1997 (29.05.97), esp. page 4, line 24 - line 36; page 10, line 18 - line 24; page 24, line 33 - line 36	1-23
X	WO 9507463 A1 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK), 16 March 1995 (16.03.95), page 15, line 11 - page 16, line 3	1-23

☒ Further documents are listed in the continuation of Box C.

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Date of the actual completion of the international search

Date of mailing of the international search report

25 May 1999

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 99/00041

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9525812 A2 (SANDOZ LTD. ET AL), 28 Sept 1995 (28.09.95), esp. page 4, line 10 - line 24  -----	1-23

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

03/05/99

International application No.

PCT/DK 99/00041

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9718841 A1	29/05/97	AU 4285096 A EP 0861093 A	11/06/97 02/09/98
WO 9507463 A1	16/03/95	AU 694745 B AU 7795794 A CA 2169298 A EP 0759170 A JP 9505981 T US 5491084 A	30/07/98 27/03/95 16/03/95 26/02/97 17/06/97 13/02/96
WO 9525812 A2	28/09/95	AU 1894995 A CA 2179890 A EP 0750681 A GB 9405350 D JP 9510355 T	09/10/95 28/09/95 02/01/97 00/00/00 21/10/97

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